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## Ex Vivo T Cell Cytokine Expression Predicts Survival in Patients with Severe Alcoholic Hepatitis

Ashwin D Dhanda<sup>1,2</sup>, Euan Yates<sup>2</sup>, Lauren P. Schewitz-Bowers<sup>3</sup>, Philippa J. Lait<sup>3</sup>, Richard W.J. Lee<sup>3</sup>, and Matthew E. Cramp<sup>1,2</sup>

<sup>1</sup>South West Liver Unit, University Hospitals Plymouth NHS Trust, <sup>2</sup>Hepatology Research Group, University of Plymouth, Plymouth, and <sup>3</sup>Translational Health Sciences, University of Bristol, Bristol, UK

Alcoholic hepatitis (AH) is an acute inflammatory liver condition with high early mortality rate. Steroids improve short-term survival but nonresponders have the worst outcomes. There is a clinical need to identify these high-risk individuals at the time of presentation. T cells are implicated in AH and steroid responsiveness. We measured ex vivo T cell cytokine expression as a candidate biomarker of outcomes in patients with AH. Consecutive patients (bilirubin >80  $\mu\text{mol/L}$  and ratio of aspartate aminotransferase to alanine aminotransferase >1.5 who were heavy alcohol consumers with discriminant function [DF]  $\geq 32$ ), were recruited from University Hospitals Plymouth NHS Trust. T cells were obtained and stimulated ex vivo. Cytokine expression levels were determined by flow cytometry and protein multiplex analysis. Twenty-three patients were recruited (10 male; median age 51 years; baseline DF 67; 30% 90-day mortality). Compared to T cells from nonsurvivors at day 90, T cells from survivors had higher baseline baseline intracellular interleukin (IL)-10:IL-17A ratio (0.43 vs 1.20,  $p=0.02$ ). Multiplex protein analysis identified interferon  $\gamma$  (IFN $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) as independent predictors of 90-day mortality ( $p=0.04$ ,  $p=0.01$ , respectively). The ratio of IFN $\gamma$  to TNF- $\alpha$  was predictive of 90-day mortality (1.4 vs 0.2,  $p=0.03$ ). These data demonstrate the potential utility of T cell cytokine release assays performed on pretreatment blood samples as biomarkers of survival in patients with severe AH. Our key findings were that intracellular IL-10:IL-17A and IFN $\gamma$ :TNF- $\alpha$  in culture supernatants were predictors of 90-day mortality. This offers the promise of developing T cell-based diagnostic tools for risk stratification. (**Gut Liver, Published online November 14, 2019**)

**Key Words:** Hepatitis, alcoholic; T cells; Cytokines; Biomarker

### INTRODUCTION

Alcoholic hepatitis (AH) is an acute inflammatory condition, which carries a high mortality of 30% within 90 days.<sup>1</sup> Steroid treatment is the only therapy with proven short-term survival benefit.<sup>2</sup> However, the worst outcomes are in the 30% to 40% of patients with a poor response to steroid treatment,<sup>3</sup> and there is a pressing need to identify these high-risk individuals at presentation.

T cells have long been implicated in the pathogenesis of AH and we have previously reported that suppression of lymphocyte proliferation correlates with clinical outcome following steroid treatment.<sup>4,5</sup> Given the barriers to the development of this radiation-based assay for clinical application and the precedent of functional cytokine release assays for other clinical applications,<sup>6</sup> we sought to evaluate T cell cytokine expression as a candidate biomarker of AH mortality.

### CASE REPORT

#### 1. Study design

The study protocol was approved by the UK's Health Research Authority (reference: 15/LO/1501) and all participants provided written informed consent. Consecutive patients with severe AH, defined as recent onset jaundice with bilirubin >80  $\mu\text{mol/L}$  and ratio of aspartate aminotransferase to alanine aminotransferase >1.5 in heavy alcohol consumers (>60 g or 80 g ethanol/day in females and males respectively) with a discriminant function (DF) score  $\geq 32$ , were recruited from University Hospitals Plymouth NHS Trust. Patients received standard care including steroid treatment in the absence of active infection, hepatorenal syndrome or gastrointestinal hemorrhage.

Correspondence to: Ashwin D Dhanda

South West Liver Unit, University Hospitals Plymouth NHS Trust, Plymouth PL6 8DH, UK

Tel: +44-1752-432723, Fax: +44-1752-517576, E-mail: ashwin.dhanda@plymouth.ac.uk

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## 2. T cell isolation, stimulation and statistical analysis

Whole blood samples were taken before steroid treatment was started with CD4<sup>+</sup> T cells isolated by negative selection (Stemcell Technologies, Cambridge, UK) and cultured for 4 days in supplemented media with interleukin (IL)-2 (Sigma-Aldrich, Poole, UK) and T cell receptor stimulation (anti-CD3/CD28 microbeads; Thermo Fisher Scientific, Loughborough, UK). Cells cultured without T cell receptor stimulation were included as controls. T cell receptor stimulation with anti-CD3/CD28 microbeads was selected as a standardized method of T cell activation, which has been optimized for use in other conditions.<sup>7</sup> For the final 4 hours, cultures were stimulated with T cell mitogen phorbol 12-myristate 13-acetate and ionomycin (Sigma-Aldrich) with Golgi export inhibitor (BD Biosciences, Oxford, UK). Cells were fixed, permeabilized and stained with fluorescently labelled antibodies to IL-10, IL-17A and interferon  $\gamma$  (IFN $\gamma$ ) (Thermo Fisher Scientific), quantified on a BD Accuri flow cytometer (BD Biosciences) and analyzed in FlowJo (FlowJo LLC, Ashland, OR, USA). Protein concentration in cell culture supernatants prior to the final 4-hour stimulation was analyzed in duplicate for CCL20, granulocyte-macrophage colony stimulating factor, IFN $\gamma$ , IL-10, IL-12p70, IL-17A, IL-21, IL-23, IL-4, IL-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) using a magnetic bead array (R&D Systems, Minneapolis, MN, USA) on a Luminex 200 analyzer (Luminex Corp, Austin, TX, USA). Statistical analysis was performed using IBM SPSS version 24 (IBM Corp., Armonk, NY, USA). The primary clinical outcome was death within 90 days of presentation. The data presented are median values and comparisons were made with nonparametric tests.

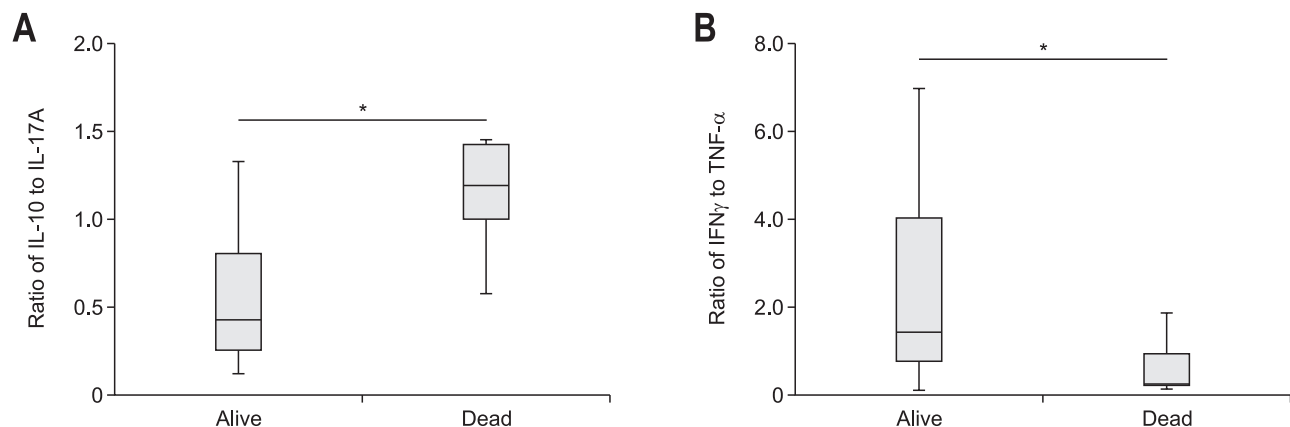
## 3. Findings

Twenty-three consecutive patients were recruited between April 2016 and November 2017 (10 male; median age, 51 years; baseline DF, 67; Model for End-Stage Liver Disease [MELD]

score, 19). Ninety-day mortality was 30%. Four patients did not receive steroids because of active infection. Samples were obtained prior to steroid treatment in all cases at a median of 5 days from hospital admission. Median time from admission to steroid treatment was 6 days.

Compared to survivors at day 90, CD4<sup>+</sup> T cells from nonsurvivors had a higher baseline intracellular IL-10:IL-17A ratio (percent of T cells expressing IL-10: 2.4% [nonsurvivors] vs 1.6% [survivors],  $p=0.07$ ; IL-17A: 2.1% [nonsurvivors] vs 3.5% [survivors],  $p=0.08$ ; median IL-10:IL-17A ratio: 1.20 [nonsurvivors] vs 0.43 [survivors];  $p=0.02$ ) (Fig. 1A) with an area under the curve of the receiver operating characteristic score of 0.82 (95% confidence interval, 0.64 to 1.00). The proportion of T cells expressing IFN $\gamma$  was similar between groups (14.2% vs 11.9%,  $p=0.55$ ). In comparison, expression of cytokines in control cultures was lower than stimulated conditions: IL-10: 0.2% vs 1.8%; IL-17: 2.5% vs 3.3%; IFN $\gamma$ : 6.2% vs 13.3% (all  $p=0.07$ ; Wilcoxon signed ranks test).

Multiplex protein analysis performed on 22 of the 23 cases identified IFN $\gamma$  and TNF- $\alpha$  as most differentially expressed between survivors and nonsurvivors at day 90 (7,044 pg/mL vs 2,741 pg/mL,  $p=0.19$  and 2,610 pg/mL vs 4,098 pg/mL,  $p=0.08$ , respectively). Multivariate analysis confirmed that both IFN $\gamma$  and TNF- $\alpha$  were independent predictors of 90-day mortality ( $p=0.04$  and  $p=0.01$ , respectively). The ratio of IFN $\gamma$  to TNF- $\alpha$  was predictive of 90-day mortality (1.4 vs 0.2,  $p=0.03$ ) (Fig. 1B) with an area under the curve of the receiver operating characteristic score of 0.79 (95% confidence interval, 0.58 to 0.99). The concentration of other mediators tested in the multiplex was similar between survivors and nonsurvivors. No cytokine concentration correlated with baseline DF or MELD scores. Steroid response, as determined by Lille score with a threshold of 0.45 at day 7 of steroid treatment, was not associated with differences in any cytokine measured by either flow cytometry or protein multiplex. Similarly, the ratios of intracellular IL-10/IL-



**Fig. 1.** (A) The ratio of the percentage of CD4<sup>+</sup> T cells expressing IL-10 to IL-17A measured by flow cytometry (0.4 vs 1.2,  $p=0.02$ ) and (B) the ratio of the IFN $\gamma$  to TNF- $\alpha$  concentrations measured by protein multiplex in patients with AH who were survivors and nonsurvivors at day 90 (1.4 vs 0.2,  $p=0.03$ ).

IL, interleukin; IFN $\gamma$ , interferon  $\gamma$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . \* $p<0.05$ .

17A and IFN $\gamma$ /TNF- $\alpha$  were the same in Lille steroid responders and nonresponders.

## DISCUSSION

These data from a pilot prospective cohort of more than 20 patients demonstrate the potential utility of T cell cytokine release assays performed on pretreatment blood samples as biomarkers of survival in severe AH. Our key findings were that both the ratio of intracellular IL-10 to IL-17A measured by flow cytometry in CD4<sup>+</sup> cells and the ratio of IFN $\gamma$  to TNF- $\alpha$  in culture supernatants were predictors of 90-day mortality. These data also demonstrate that flow cytometry and protein multiplex are complementary techniques that can reveal different alterations in protein expression either in proportion of cells secreting a cytokine or in cytokine concentration, respectively.

The pathogenesis of AH is orchestrated by several immune subsets including neutrophils and monocytes, which are activated by gut derived signals such as endotoxin.<sup>8,9</sup> However, T cells also play a vital role in responding to antigen presenting cells and propagating inflammation in AH.<sup>10</sup> Transcriptome studies have confirmed upregulation of TNF and T cell pathways.<sup>11,12</sup> In particular, the Th17 (IL-17 expressing T cell) pathway is up-regulated with high expression of related chemokines such as CCL20.<sup>12,13</sup> Furthermore, recent studies have demonstrated that monocytes activated by exposure to gut microbial signals interact with CD4<sup>+</sup> T cells to alter expression of exhaustion markers and cytokines including IFN $\gamma$ .<sup>14</sup> These data confirm the relevance of T cells in AH and support the measurement of T cell cytokines as a broad measure of immune response.

These findings offer the promise of developing T cell based diagnostic tools for risk stratification both in the context of routine clinical practice and also to inform the design of smart clinical trials seeking to test new interventions in patients most likely to fail conventional corticosteroid therapy.

## CONFLICTS OF INTEREST

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L.P.S. and R.W.J.L. are named inventors on a US patent application (number: 61/919,404), which incorporates biomarkers to identify patients who will benefit from treatments for inflammatory diseases.

## AUTHOR CONTRIBUTIONS

Study concept: A.D.D., R.W.J.L. Performed experimental work: E.Y., L.P.S., P.J.L. Statistical analysis: A.D.D., P.J.L. Study supervision: R.W.J.L., M.E.C. Drafting manuscript: A.D.D., L.P.S. Finalizing manuscript: A.D.D., R.W.J.L., M.E.C.

## ORCID

Ashwin D Dhanda	<a href="https://orcid.org/0000-0002-0523-0193">https://orcid.org/0000-0002-0523-0193</a>
Euan Yates	<a href="https://orcid.org/0000-0003-3082-2972">https://orcid.org/0000-0003-3082-2972</a>
Lauren P. Schewitz-Bowers	<a href="https://orcid.org/0000-0001-6559-2228">https://orcid.org/0000-0001-6559-2228</a>
Philippa J. Lait	<a href="https://orcid.org/0000-0001-9454-8824">https://orcid.org/0000-0001-9454-8824</a>
Matthew E. Cramp	<a href="https://orcid.org/0000-0002-1152-0568">https://orcid.org/0000-0002-1152-0568</a>

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